Swami Ramanand Teerth Marathwada
University, Nanded, Maharashtra, India
(UGC recognized,NAAC Re-accrediated 'B++'grade state university)

"STUDIES ON ROLE OF PHYTOTERPENOIDS ON AFLATOXIN PRODUCTION IN ASPERGILLUS PARASITICUS"

Research Proposal submitted
In partial fulfillment of registration to
Ph. D. Programme

In the subject
Microbiology
Faculty of Science

Submitted By
Shantanu Shashikant Kulkarni

Under the Supervision of
Dr. S. M. More
P. G. Dept. & Research Centre
Dept. of Microbiology
Yeshwant Mahavidyalaya, Nanded

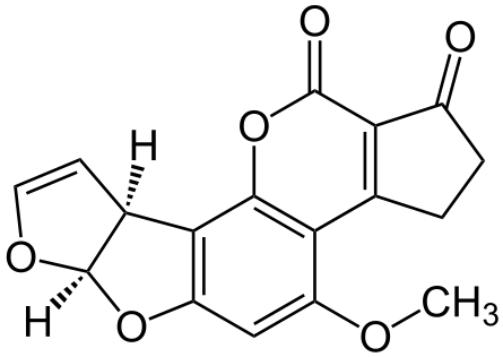
2022

1. **Research Proposal Outline:** Fungi able to grow on various types of food and feed material under storage conditions. Contamination of food and feed by mycotoxigenic mould cause significant losses, which is a global problem. Aflatoxin is a secondary metabolite produced mainly by *Aspergillus parasiticus* and *Aspergillus flavus*. *Aspergillus parasiticus* is one of the major contaminant in stored peanut. These fungi mainly contaminate food and now becoming part of food chain. Consumption of aflatoxin contaminated food by human beings is one of the reason in hepatocellular carcinoma. The present study undertaken to search for potential bioactive molecule from plant origin to control growth of mycotoxigenic fungi.
2. **Introduction:** *Aspergillus parasiticus* and *Aspergillus flavus* are major contaminants of food and feed materials (Claeys, 2020). These fungi elaborate secondary metabolites called as aflatoxins (AFs) which are carcinogenic, teratogenic, mutagenic and immunogenic in character and pose serious health hazards to humans, dairy animals and poultry when ingested (1). In addition, AFs also affect nutritional quality of food grains, oil seeds and pulses and viability of seeds (Zain, 2011). The AFs are also an international trade problem, restricting trade (Tola, 2016). Aspergillus parasiticus produces aflatoxin B1, B2, G1 and G2. These toxins are toxic and classified into Group 1 carcinogen produced naturally (IARC-2012).

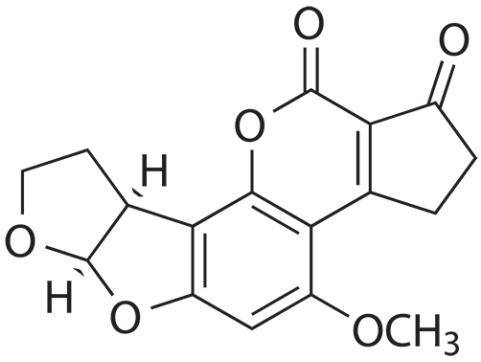
   Aflatoxins, a group of related toxic and carcinogenic bis-furan secondary metabolites, are produced primarily by the toxigenic species of

filamentous fungi belonging to *Aspergillus* genus *A. flavus, A. parasiticus, A. nomius, A. tamarii*, *A. bombysis, A. toxicarius* and *A. parvisclerotigens* (Goto et al., 1996; Peterson et al., 2001, Holmes et al., 2008) that occur in nuts, tree nuts, pea nuts, corn, cotton, cotton seeds, coffee beans and cereal crops (Varga et al., 2010, Bokhari and Aly, 2009). Aflatoxins of greater toxicological significance are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2). Aflatoxin M1 (AFM1) and aflatoxin (AFM2) are the metabolites produced primarily in the liver of exposed animals; AFM1 is produced by hydroxylation of AFB1 while AFM2 is produced by the hydroxylation of AFB2 (Bianco, G. 2012). Contamination of crops such as corn, cotton, peanuts, and tree nuts by aflatoxins represents a significant global risk to human and animal health and can cause devastating economic losses due to strict regulatory limits placed on dissemination of contaminated products (Liu and Wu, 2010). These fungi invade agricultural commodities such as corn, peanuts, and cottonseed. Colonization of maize by *Aspergillus flavus* Link and *A. parasiticus* Speare has often resulted in aflatoxin accumulation at harvest (Wilson and Payne, 1994; Resnik et al., 1996; Torres et al., 1997). The resulting contamination with aflatoxin often makes these commodities unsuitable for human and animal consumption. Since the discovery of aflatoxins in the early 1960s, numerous studies have focused on the ecology of *A. flavus* in the agroecosystem (Wicklow et al., 1998; Abbas et al., 2008; Accinelli et al., 2008). Some of the crops most susceptible to

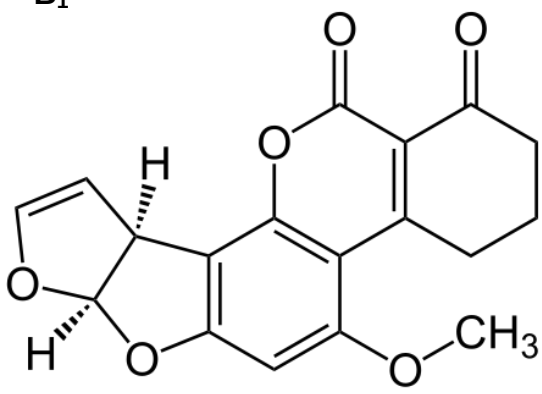
aflatoxin contamination include peanuts, cotton and corn (Scheidegger and Payne, 2003). The severity of aflatoxin contamination is largely determined by the environment, with pre-harvest contamination being favored under drought and temperature stress (Payne, 1992, 1998; McGee et al., 1996; Abbas et al., 2007). *A. flavus* is a ubiquitous fungus readily isolated from diverse environments, with soil and plant tissues or residues being the natural habitat of this fungus (Geiser et al., 2000; Horn, 2003; Abbas et al., 2009). Since soil serves as a reservoir for primary inoculums for the infection of susceptible crops, most studies have focused on the occurrence and quantification of Aspergilli propagules in soil (Horn and Dorner, 1998; Zablotowicz et al., 2007; Accinelli et al., 2008). Human and animals are exposed to aflatoxins mainly through the consumption of contaminated food and feeds, particularly products of vegetable origin such as cereal grains (Bianco, G. 2012). These compounds have a high acute toxicity, as well as potent immunosuppressive, mutagenic, teratogenic, carcinogenic, hepatotoxic, also inhibit several metabolic systems activities. (Peraica, M. 1999). AFB1 is the most potent natural hepatocarcinogen for different animal species including human, classified by the international agency for Research on cancer (IARC) in Group 1B (IARC 2002).



B_1



B_2



M_1

Among different metabolites produced in mammals following the biotransforming reactions such as AF-8-9-epoxide, aflatoxicol and AFQ1, AFM1 and AFM2 are of great concern regarding the public health because these are excreted into milk and persist in dairy products where they link to casein proteins (Bianco, G. 2012).

Aflatoxin M1 (AFM1)

AFM1 is a metabolite produced primarily in the liver of exposed animals; AFM1 is produced by hydroxylation of AFB1, excreted into milk and

persist in dairy products where link to casein proteins. The human breast milk represents another way of elimination of AFM1 and AFM2. The contamination of milk and derived products by AFM1, sometimes above the maximum limit (50 ng/kg) established by European Reg. 1881/2006. AFM1 shows a toxicological profile similar to AFB1 but it is less toxic (about 10%) than the carcinogenicity (Bianco, G. 2012).

Effect of Aflatoxin on Immune system: The toxic effects of aflatoxin on immune system have been reported. In vitro and in vivo studies have shown that AFB1 is immunotoxic, in particular, it exerts its action on cell-mediated immunity by reducing the circulating lymphocytes, inhibition of lymphocytes blastogenesis in several animal species, alteration of the activity of NK cells and expression of cytokines. In vitro studies shown that AFB1 inhibited NO production in murine macrophages, phagocytosis in alveolar macrophages of pigs and the ability to present antigen to lymphocytes.

Aflatoxins are food contaminants that can exert their toxicity representing health risk both for humans and animals, including the suppression of immunity and an increase of susceptibility to disease as the development of cancers. Among different metabolites aflatoxin 8-9-epoxide which is the most unstable metabolite able to interact with macromolecules inside the cells, two hydroxylated metabolites of the parent compounds indicated as AFM1 and AFM2 are of major concern regarding the food safety b on ecause they are excreted in milk and persist in dairy products. Food and

feed commodities are often contaminated by more than one mycotoxin, which may act as antagonists. AFB1 shows anti-proliferative activity on J774A.1 macrophages. AFB1 induces cell cycle delay by activating a DNA chectkpoint response to DNA damage or replication blocks. AFB1 induced a concentration-dependent cell accumulation in S phase in a p53-deficient human cell line (Bianco, G. 2012).

3. **Purpose:**
    - To Search for alternative natural preservatives for storage of food and feed.
    - To establish correlation between morphogenesis and aflatoxin production.
4. **Review of Literature:** The prevention of synthesis of mycotoxin in food is better, chemical options are available to tackle grain/seed contamination. Chemical pesticides like organo chlorine/phosphorus pesticides are used as seed dressers. However these are not advisable on food grains because of their toxicity to the users and the environment (Loi 2020). Most of the agricultural commodities like, peanut, maize, chilly are vulnerable to contamination by *Aspergillus sps*. (Fouad et al. 2019). Sorghum [*Sorghum bicolor* (L.) Moench] is one of the main staples for the world's poorest and most food insecure people. Sorghum is grown in kharif (rainy), rabi (post rainy) and summer seasons in India. Sorghum is a truly dual-purpose crop; both grain and stover are highly valued outputs. Grain mold on sorghum is an important disease worldwide, which causes

considerable qualitative and quantitative damage (Audilakshmi et al., 2011). Grain molds cause significant losses in both grain yield and its nutritional quality. Sorghum is the preferred staple food in states of Maharashtra, Karnataka and Andhra Pradesh in India. Sorghum grain grown in kharif is severely affected by grain molds due to heavy rains at the time of crop maturity. If the extent of mold is severe the grain is unsafe for consumption due to the contamination of mycotoxins. Grain sorghum is often damaged by rain in the field and severely infected by grain mold, which includes Aspergillus infection and aflatoxin production (Ratnavathi & Sashidhar, 2003). Mycotoxin contamination and grain mold of sorghum are considered as the most important constraints of grain quality and production, globally. Sorghum grains suffer from infection and colonization by several fungi during panicle and grain developmental stages (Waliyar et al., 2008, p. 32). Several species of *Aspergillus, Alternaria, Cladosporium, Diplodia, Fusarium, Curvularia, Phoma* and *Penicillium* are among the prevalent grain mold pathogens in sorghum (Bandyopadyay, Butler, Chandrashekar, Reddy, & Navi, 2000). The natural occurrence of aflatoxin B1in rain-affected Indian sorghum samples and aflatoxin B1 contamination in Brazilian samples were reported by Silva, Pozzi, Mallozzi, Ortega, and Correa (2000). Sashidhar, Ramakrishna, and Bhat (1992) reported a systematic study on the mold and mycotoxin contamination in the grain sorghum stored in traditional containers in India. The improvement in sorghum grain quality occurs when the crop

was harvested at physiological maturity and artificial drying (Audilakshimi et al., 2005). Grain mold resistance at physiological maturity is genetically governed and the grain mold score further gets compounded at harvest maturity depending on (environment) rainfall received after physiological maturity (Ambekar et al., 2011). Acetic acid treatment was most effective for reducing the grain mold score in sorghum (Audilakshimi et al., 2007).Fungal deterioration of stored grains is a chronic problem in the Indian storage system because of the tropical hot and humid climate. Infection of *Aspergillus* spp. was found on most sorghum grains collected from different sorghum growing areas in India either at kharif season or during storage.

There is a necessity for nontoxic and ecofriendly seed dressers or preservatives of food/feed grains and seed materials. Many of the spices, essential oils and their components which are traditionally used in food as flavoring agents possess antifungal properties (da Silva Bomfim et al. 2020).Various workers have reported their efficacy against fungal growth. For example, the anti-aspergillus properties of essential oils of clove, cinnamon and holy basil (*Ocimum sanctum*) are well documented (Esper et al. 2014). Some of the major ingredients of clove and cinnamon, eugenol and cinnamaldehyde can inhibit growth, sporulation and aflatoxin production by *A. parasiticus* and *A. flavus* (Nazzaro et al. 2017). A number of plants and their extracts are investigated for their antifungal properties often inspired by their traditional uses (Raveau et al. 2020). Inspite of the

vast literature on the antifungal activities of the spices, plant based products are yet to enter in to the commercial pesticide market. The potential of using neem oil (*Azadirachata indica*) as a preservative against *Aspergillus* contamination was reported (Costa et al. 2010). The safe limit of aflatoxin lies between 4-30 ug/kg for human consumption. The European Union has the strictest standard level with AFB1 and total AFs not beyond 2ug/kg and 4ug/kg respectively in any product meant for direct consumption (Mahato et al 2019).

5. **Research gap(s):** Majority of previous study reported inhibition of aflatoxin in liquid conditions. The storage conditions of food and feed are in solid condition we are focusing on conducting research in solid condition so that use of natural products will be effective

6. **Importance of the study:**

   International Status: The aflatoxin producing fungi usually infect cereal crops including wheat, walnut, corn, cotton, peanuts, millet, sesame seeds, maize, rice, fig, spices and cocoa. The presence of aflatoxin also reported in commercial products like peanut butter, cooking oil and cosmetics (Mahato et al 2019)

   National Status: One of the first outbreaks of aflatoxicosis was reported form Banswara (Rajasthan) and Panchmahals (Gujarat) India during 1975 with 106 deaths of tribal group of Bhils due to consumption of contaminated maize. Indian childhood cirrhosis, a clinical condition mainly confined to the Indian subcontinent has been attributed to aflatoxin

contamination. The correlation between aflatoxin contamination and hepatomegaly in children in south Canara district of Karnataka was reported. Heavy mortality in chicks in Cittoor district of Andhra Pradesh was reported in 1982 due to aflatoxicosis. A case of aflatoxicosis in Murrah buffaloes from Andhra Pradesh was also reported. Disease outbreaks due to aflatoximans continue to be problems of significant public health concern in India as long as people will consume contaminated food (Reddy et al 2007).

Regional Status: Maharashtra is the 6th largest groundnut producing state in the country. The maize production in Maharashtra contributes 10% of total production (Mahato et al 2019). Peanut and maize both are important food and consumed daily. The losses of peanut and maize during storage can be minimized by our study.

Scope of study: Different strategies are used to prevent production of aflatoxin by fungi in food commodities. These methods include good agricultural practices to the use of biocontrol agents to prevent production of aflatoxin. The use of natural product to control growth of mycotoxigenic mould is having scope of research. Therefore studies adding more information on effect of natural plant components in different aflatoxigenic fungi still required.

7. **Objectives:**

- To isolate fungi from peanut.

- To screen potential molecules of plant origin as antiaspergillus agents.
- To study relationship between sporulation and aflatoxin biosynthesis.
- To screen of molecules of plant origin as inhibitor of sporulation and aflatoxin biosynthesis.
- To study effect of plant molecules on morphological development of *Aspergillus spp.*
- To study the effect of plant molecules on seed germination.
- To study effect of plant molecules on radial growth of *Aspergillus spp*.
- To study the status of sporulation under aflatoxin inhibition conditions and vice versa.

8. **Hypothesis:** Plant components are effective against growth, sporulation and aflatoxin production in *Aspergillus parasiticus* and can be used as seed dressers.
9. **Materials and Methods:**

Chemicals used: All the chemicals used were of Analytical Grade

Cultures: The fungal strains used in this study will be *Aspergillus parasiticus* var.*globosus* Murakami ATCC15517 (MTCC411), *Aspergillus parasiticus* speare SRRC1008 (MTCC2797), *Aspergillus flavus* Link NRRL18079 (MTCC2798). All strains are known to elaborate aflatoxins and will be obtained from the Institute of Microbial Technology, Chandigarh, India.

Isolation of Fungi: The isolation of fungi from contaminated peanut will be carried out and identification of fungi by microscopic and mycotoxin production ability.

Determination of Spore Number: The strains were cultivated on PDA slants at 30ºC until they sporulate. Spores were flooded with 10 ml of sterile distilled water containing 0.05 % Tween 80. Spores were gently collected and resulting suspension was mixed thoroughly with a vortex mixer. This spore suspension was diluted an inoculum concentration of 10^7 Spores mL^{-1}. Spore number was determined using Neubauer Haemocytometer (Hitokoto et al. 1980).

Growth of *Aspergillus parasiticus* and *Aspergillus flavus* in presence of Plant Components: Minimum Inhibitory Concentration (MIC) of plant components were determined by using Broth dilution technique (Sydney et al. 1978). Tests were performed in Yeast Extract Sucrose broth of pH 6.0 (Yeast Extract 2%, Sucrose 15%). Plant components were dissolved in (1:1 v/v) ethanol. Different concentrations of plant components ranging from 3.2% - 0.031% (v/v) will be prepared in 2mL broth with final concentration of 0.001% Tween 80. For each concentration, three tubes were used. Growth controls (without plant components), sterility controls (without plant components and uninoculated) and solvent controls (with highest and lowest concentrations of ethanol) will be kept in triplicates. These tubes will be inoculated with ~10^5 Spores mL^{-1} of spore suspension of test organisms. The tubes will be incubated at 30ºC for 72 hours. After every 24 hours each tube was vortexed

for 10s and presence of growth was compared with controls. The lowest concentration of plant component inhibiting complete fungal growth was considered as MIC (Sydney et al. 1978).

Effect of Plant components on Growth: Different concentrations of plant components i.e. 0.0031, 0.0062, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 will be prepared in Erlenmeyer flasks containing sterile medium (Yeast Extract Sucrose Agar) with 0.001% Tween 80. Flask was shaken gently and 20 mL of medium will be dispensed in sterile petriplates and allowed to solidify. A well was carved in the center of solidified plate with a sterile cork borer and 10µl aliquot (~10^3 spores) of spore suspension will be placed. Plates without plant components will be kept as negative control. Plates were incubated at 30º C. Radial growth diameter will be measured after regular intervals of 24 hours. Percentage inhibition of growth is calculated using formula Percent Inhibition = (C–T/C) × 100 (C = Area of radial growth in control, T = Area of radial growth in treatment with eugenol). For each concentration, plates were kept in triplicates and experiment was repeated three times. The average values of three experiments were taken (Guzman-de-Pena D. 1997).

Sporulation of *Aspergillus parasiticus* and *Aspergillus flavus* in presence of plant components: Three plates with different concentration of plant components and control were removed after regular interval of 24 hours. Spores were flooded with 10 ml of sterile distilled water containing 0.05 % Tween 80. Spores were gently collected and resulting suspension was mixed

thoroughly with a vortex mixer. Spore number was determined using Neubauer Haemocytometer. The number of spores / cm^2 of area of growth were calculated. Percentage inhibition of sporulation was calculated using formula Percent Inhibition = (C–T/C) × 100 (C = Sporulation /cm^2 in control, T = Sporulation /cm^2 in treatment with eugenol) (Hitokoto et al. 1980; Guzman-de-Pena and Ruiz-Herrara 1997).

Aflatoxin Production in Presence of Plant components: Aflatoxin extraction was carried out after 96 hours using solvent extraction procedure with some modifications. Agar medium with growth was weighed on single pan balance and transferred in Mortal and Pestle. Sodium chloride (80mg/gm) was added and agar was crushed and transferred to a Erlenmeyer flask containing 50mL chloroform. Flasks were plugged tightly, wrapped with a black paper and kept on an orbital shaker at 120 rpm for 1-1.30 hours. The chloroform fraction was filtered and collected in a beaker. Chloroform was evaporated by keeping it on a boiling water bath. Dried chloroform extract was reconstituted in Benzene: Acetonitrile (98:2), collected in Eppendroff tube (1.5mL) and volume was made to 1mL. This extract was stored at 4º C until further analysis (Pallavi et al. 1997).

Analysis of Aflatoxins: 10×10cm aluminium precoated silica gel 60 F_{254} Thin Layer Chromatography (TLC) plates (Merck, Darmstadt Germany) will be used for aflatoxin chromatography. For each concentration of plant components three sets of experiments were carried. Dried chloroform extracts were reconstituted in Benzene: Acetonitrile in such a way that growth

area/µl was uniform to all concentrations. The samples were applied along with reference standards using a semiautomatic sample applicator, Linomat5 (Camag, Switzerland). Plates were developed with Toluene: Ethyl Acetate: Formic Acid (6:3:1) as solvent system. Densitometric scanning of thin layer chromatograms was carried out using a TLC Scanner3 (Camag Switzerland) at 366 nm. The quantification was done using win CATS Planar Chromatography Manager (Camag Switzerland). The percentage inhibition of aflatoxin was carried out by formula Percent Inhibition = (C–T/C) × 100 (C = Aflatoxin in control, T = Aflatoxin in treatment with eugenol) (Pallavi et al. 1997).

Conidial Morphogenesis in *Aspergillus parasiticus* and *Aspergillus flavus* in presence of Plant components: One round piece of filter paper was placed in bottom of Petri dish and moistened. The petriplates were sterilized. Two applicator sticks were placed aseptically using a flamed forceps. A clean slide, sterilized by flaming was placed across applicator sticks. A 10 mm cut of agar block was cut using cutter and placed on slide. About 100 spores were inoculated on each of the four corner of the agar block. Sterile cover slip was placed on surface of agar block. Plates were incubated at 30º C. Four concentrations of each plant component were tested. For each concentration three agar blocks were used. Solvent controls with respective concentration of ethanol were also tested. Negative controls with no plant components or solvent were used. Observations were carried out using high power objective (400X) after 24hrs by using Labomed ATC

2000 Compound microscope. Photographs were captured using CCD digital camera (Aneja 2003)

Effect of Plant components on seed germination of Peanut seeds: Peanut seeds (*Arachis hypogea*) without any antifungal treatment were purchased from the local market. 100 numbers of healthy seeds were randomly selected and washed 2-3 times with sterile distilled water. Different concentrations of plant components were prepared by diluting (1:2) with ethanol (S.D. Fine Lab. Ltd. Mumbai). 100 ml sterile distilled water used for treatment of 100 seeds. Concentrations of plant components will be adjusted by addition in distilled water with 0.01% Tween 80. The seeds will be kept for treatment in a beaker for seven min and again washed with sterile distilled water. Three controls i.e. sterile distilled water, ethanol, and sodium hypochlorite were used and seeds were treated similarly. A blotting paper (40cm x 25 cm) soaked with sterile distilled water was spread on plain platform. Treated seeds were kept at a distance of 5cm in rows of five seeds in each row. Another blotting paper soaked in sterile distilled water was spread over seeds and a paper roll of for each concentration of eugenol was prepared. The rolls were kept in clean perforated plastic boxes. Germination of peanut seeds were recorded in terms of root and shoot length after intervals of 24 hours up to seven days (AOSA 1981).

Statistical Analysis: For each concentration of plant components tested plates were kept in triplicates and experiment was repeated three times. The

most significant values were given in table from mean of three values. Standard deviation will be calculated.

10. **Limitation(s):** The study includes use of invitro methods or strategies to prevent growth of fungi. The study at large scale need to be performed to draw final conclusions.

11. **Expected outcome:**

- We are screening for about ten natural products they all may be used as good seed treatment agent in storage conditions.
- Relationship between growth sporulation and aflatoxin production will be established.

12. Proposed work plan

Sr. No.	Proposed Work Plan	Time Required
1	Extensive review on pervious work	6 Months
2	Isolation of fungi and Screening of Plant components	6 Months
3	Studies on effect of plant molecules on growth of *Aspergillus parasiticus*	6 Months
4	Studies on effect of plant molecules on sporulation of *Aspergillus parasiticus*	6 Months
5	Studies on morphogenesis of *Aspergillus parasiticus*	6 Months
6	Studies on effect on plant molecules on aflatoxin production by *Aspergillus parasiticus*	6 Months
7	Studies on effect of plant molecules on germination of seed mainly pea nut	6 Months

13. Year wise plan of work and targets to be achieved

Year	Targets to be achieved
1	Finding of effective plant components against Aspergillus parasiticus
2	Finding of effect of plant components on Growth, Sporulation and aflatoxin production. Communication with journals to publish the findings
3	Findings on use of plant molecules as seed dresser. Publish the findings
4	Submission of the thesis

14. References

1. Abdel-Hadi, A. M., Caley, D. P., Carter, D.R.F., Magan, N. 2011. Control of Aflatoxin Production of *Aspergillus flavus* and *Aspergillus parasiticus* Using RNA Silencing Technology byTargeting *aflD* (nor-1) Gene. Toxins : 3: 647-659
2. Accinelli, C., Mencarelli M., Ludovica Sacca M., Vicari A., Abbas H. K. 2012. Managing and monitoring of *Aspergillus flavus* in corn using bioplastic-based formulations. Crop Protection 32: 30-35
3. Ambekar, S. S., Kamatar, M. Y., Ganeshmurthy, K., Ghorade, R. B., saxena, U., Chand, P., et al. 2011. Genetic enhancement of Sorghum (Sorghum bicolor (L.) Moench for grainmould resistance: II. Breeding for grain mould resistance. Crop Protection, 30, 759-764.

4. Aneja, KR (2003) (Microscopic examination of living microorganisms In: Experiments in Microbiology, Plant Pathology and Biotechnology, 4th edn, New Age International (P) Limited, New Delhi, pp 83-85.
5. Association of official seed analysis (AOSA) (1981) Rules for testing seeds. J Seed Technol.6:1-125.
6. Audilakshimi, S., Aruna, C., Garud, T. B., Nayakar, N. Y., Atale, S. B., Veerabadhriran, P., et al. (2005). A technique to enhance the quality and market value of rainy season sorghum grain. Crop Protection, 24,251-258.
7. Audilakshmi, S., Das, I. K., Ghorade, R. B., Mane, P. N., Kamatar, M. Y., Narayana, Y. D., et al. (2011). Genetic improvement of sorghum for grain mould resistance: I performance of sorghum recombinant inbred lines for grain mould reactions across environments. Crop Protection, 30, 753-758.
8. Bandyopadyay, R., Butler, D. R., Chandrashekar, A., Reddy, R. K., & Navi, S. S. 2000. Biology, epidemiology and management of sorghum grain mold. In A. Chandrashekar, R. Bandyopadhyay, & A. J. Hall (Eds.), Technical and institutional options for sorghum grain mold management: Proceedings of an international consultation (pp. 34-71). Patancheru 502324, Andhra Pradesh, India: International Crops Research Institute for Semi-Arid Tropics.
9. Basilico MZ, Basilico JC 1999. Inhibitory effects of some spices essential oils on *Asperillus ochraceus* NRRL3174 growth and ochratoxin A production. Lett. Appl. Microbiol. 29 (4): 238-241.

10. Buchanan RL, Lewis DF 1984. Caffiene inhibition of aflatoxin synthesis: proble site of action. App. Environ. Microbiol. 47(6): 1216-1220.
11. Bokhari, F. and Aly, M. M. 2009. Trials towards reduction of fungal growth and aflatoxin G1 production in Arabic coffee using different additives. African Journal of Food Science. 3. 068-076.
12. Champe SP, El Zayat AAE 1989. Isolation of a sexual sporulation hormone from Aspergillus nidulans. J. Bacteriol. 171: 3982-3988.
13. Claeys, L.; Romano, C.; De Ruyck, K.; Wilson, H.; Fervers, B.; Korenjak, M.; Zavadil, J.; Gunter, M.J.; De Saeger, S.; De Boevre, M.; et al. (2020) Mycotoxin exposure and human cancer risk: A systematic review of epidemiological studies. Compr. Rev. Food Sci. Food Saf. 19, 1449–1464. [CrossRef]
14. Clevstrom G, Moller T, Goransson B, Liljensjoo A, Liunggren H 2004. Influence of folic acid on fungal flora of barley and on aflatoxin production in *Asperigillus flavus* link. Mycopatholoia. 107(2-3):101-103.
15. Costa CL, Geraldo MRF, Arroteia CC, Kemmelmeider C (2010) *In vitro* activity of neem oil [*Azadirachata indica* A. Juss (*Meliaceae*)] on *Aspergillus flavus* growth, sporulation, viability of spores, morphology and aflatoxins B1 and B2 production. Advances in Bioscience and Biotechnology 01:292-299.
16. da Silva Bomfim, N.; Kohiyama, C.Y.; Nakasugi, L.P.; Nerilo, S.B.; Mossini, S.A.G.; Ramoli, J.C.Z.; de Abreu Filho, B.A.; Machinski, M. Jr.

(2020) Antifungal and antiaflatoxigenic activity of rosemary essential oil (Rosmarinus officinalis L.) against Aspergillus flavus. Food Addit. Contam. A 37, 153–161. [CrossRef]

17. Esper, R.H.; Gonçalez, E.; Marques, M.O.M.; Felicio, R.C.; Felicio, J.D. (2014) Potential of essential oils for protection of grains contaminated by aflatoxin produced by Aspergillus flavus. Front. Microbiol. 5, 269. [CrossRef]
18. Fouad, A.M.; Ruan, D.; El-Senousey, H.K.; Chen, W.; Jiang, S.; Zheng, C. (2019) Harmful Effects and Control Strategies of Aflatoxin B1 Produced by Aspergillus flavus and Aspergillus parasiticus Strains on Poultry: Review. Toxins 11, 176. [CrossRef] [PubMed]
19. Guzman-de-Pena D, Ruiz-Herrara J (1997) Relationship between aflatoxin biosynthesis and sporulation in *Aspergillus parasiticus*. Fungal Genet Biol: 21:198-205.
20. Goto, T., Wicklow, D.T., Ito, Y., 1996. Aflatoxin and cyclopiazonic acid production by a sclerotium producing *Aspergillus tamarii* strain. Appl. Environ. Microbiol., 4036–4038.
21. Hitokoto H, Morozumi S, Wauke T, Sakai S, Kurata H (1980) Inhibitory effects of spices on growth and toxin production of toxigenic fungi. Appl Envir Microbiol 39:818-822.
22. Holmes R, Boston R, Payne G. 2008. Diverse inhibitors of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol. 78(4): 559-572.

23. IARC (International Agency for Research on Cancer). Aflatoxins. In IARC Monographs on the Evaluation of Carcinogenic Risks on Humans; IARC (International Agency for Research on Cancer): Lyon, France, 2012; Volume 100 F, pp. 225–248.
24. Jayashree, T., & Subramanyam, C. 1999. Antiaflatoxigenic activity of eugenol is due to inhibition of lipid peroxidation. Letters in Applied Microbiology, 28,179e183.
25. Juglal S, Govinden R, Odhav B., 2002. Spice oil for the control of the co-occurring mycotoxin producing fungi. J. Food Prod. 65(4): 683-687.
26. Liu, Y., Wu, F., 2010. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. Environmental Health Perspectives 118, 818–824.
27. Loi, M.; Paciolla, C.; Logrieco, A.F.; Mulè, G. (2022) Plant Bioactive Compounds in Pre- and Postharvest Management for Aflatoxins Reduction. Front. Microbiol. 2020, 11, 243. [CrossRef] J. Fungi 8, 482 19 of 22
28. Mahato D. K., Lee K. E., Kamle M., Devi S., Dewangan K. N., Kumar P. and Kang S. G. (2019) Aflatoxins in Food and Feed: An Overview on Prevalence, Detection and Control Strategies. Front Microbiol. 10:2266
29. Minto, R.E., Townsend, C.A., 1997. Enzymology and molecular biology of aflatoxin biosynthesis. Chem. Rev. 97, 2537–2555.
30. Nazzaro, F.; Fratianni, F.; Coppola, R.; Feo, V.D. (2017) Essential oils and antifungal activity. Pharmaceuticals 10, 86. [CrossRef] [PubMed]

31. Norton R. A., 1999. Inhibition of aflatoxin B1 biosynthesis in *Aspergillus flavus* by anthocyandins and related flavonoids. J. Agric. Food Chem. 47: 1230-1240.
32. Peraica, M.; Radic, B.; Lucic, A.; Pavlovic, M., 1999. Toxic effects of mycotoxins in humans. *Bull. World Health Organ*.*77*, 754–766.
33. Peterson, S.W., Ito, Y., Horn, B.W., Goto, T., 2001. *Aspergillus bombycis*, a new aflatoxicogenic species and genetic variation in its sibling species, *A. nomius*. Mycologia 93, 689–703.
34. Ratnavathi, C. V., & Sashidhar, R. B. (2003). Substrate suitability of different genotypes of sorghum in relation to *Aspergillus* infection and aflatoxin production. Journal of Agricultural Food Chemistry, 51, 3482-3492.
35. Raveau, R.; Fontaine, J.; Lounès-Hadj Sahraoui. (2020) A. Essential oils as potential alternative biocontrol products against plant pathogens and weeds: A review. Foods 9, 365. [CrossRef]
36. Reddy, Bhumi & Raghavender, Chinnam. (2007). Outbreaks of Aflatoxicoses in India. African Journal of Food, Agriculture, Nutrition and Development (ISSN: 1684-5358) Vol 7 Num 5. 7. 10.18697/ajfand.16.2750.
37. Reddy, C. S., Reddy, K. R. N., Prameela, M., Mangala, U. N., & Muralidharan, K. 2007. Identification of antifungal component in clove that inhibits *Aspergillus* spp. colonizing rice grains. Journal of Mycology and Plant Pathology, 37(1), 87e94.

38. Sashidhar, R. B., Ramakrishna, Y., & Bhat, R. V. 1992. Moulds and mycotoxins stored in traditional containers in India. Journal of Stored Production Research, 28, 257-260.
39. Schatzmayr, G., Zehner, F., Taubel, M., Schatzmayr, D., Klimitsch, A., Loibner, A. P., and Binder, E. M. 2006. Microbiologicals for deactivating mycotoxins. Molecular Nutrition Food Research, 50, 543-551.
40. Scheidegger, K.A., Payne, G.A., 2003. Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. Toxin Rev. 22, 423e459.
41. Silva, J. B., Pozzi, C. R., Mallozzi, M. B., Ortega, E. M., & Correa, B. 2000. Mycoflora and occurrence of aflatoxin B1 and fumonisin B1 during storage of Brazilian sorghum. Journal of Agricultural Food Chemistry, 48, 4352-4356.
42. Sydney MF, William JM, Elvya GS. (1978) Bailey and Scott's Diagnostic Microbiology. St. Louis, MO: Moseby. 385-403p.
43. Tola, M.; Kebede, B. (2016) Occurrence, importance and control of mycotoxins: A review. Cogent Food Agric.2, 1191103. [CrossRef]
44. Varga, J., Frisvad, J.C., Samson, R.A., 2010. A reappraisal of fungi producing aflatoxins. World Mycotoxin Journal 2, 263–277.
45. Waliyar, F., Reddy, C. R., Alur, A. S., Reddy, S. V., Reddy, B. V. S., Reddy, A. R., et al. (2008). Management of grain mold and mycotoxins in sorghum. Patancheru 502324, India: International Crops Research Institute for Semi-Arid Tropics.

46. Wilson DM, Gueldner RC, McKinney JK, Lieusay RH 1981. J. Am Chem. Soc. 58: 959.
47. Zain, M.E. (2011) Impact of mycotoxins on humans and animals. J. Saudi Chem. Soc. 15, 129–144. [CrossRef]
48. Zeringue HJ Jr, Bhatnagar D 1999. Neem and control of aflatoxin contamination. In: Singh RP, eds. Neem and Environment. New Hampshire: Science Publishers, pp. 883-893.
49. Zeringue HJ Jr, McCormick SR 1990. Aflatoxin production in cultures of *Aspergillus flavus* incubated in atmospheres containing cotton leaf derived volatiles. Toxicol 28: 445-448.